

Sequence Requirements for Premature Transcription Arrest within the First Intron of the Mouse *c-fos* Gene

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A strong block to the elongation of nascent RNA transcripts by RNA polymerase II occurs in the 5' part of the mammalian *c-fos* proto-oncogene. In addition to the control of initiation, this mechanism contributes to transcriptional regulation of the gene. In vitro transcription experiments using nuclear extracts and purified transcription templates allowed us to map a unique arrest site within the mouse first intron 385 nucleotides downstream from the promoter. This position is in keeping with that estimated from nuclear run-on assays performed with short DNA probes and thus suggests that it corresponds to the actual block in vivo. Moreover, we have shown that neither the *c-fos* promoter nor upstream sequences are absolute requirements for an efficient transcription arrest both in vivo and in vitro. Finally, we have characterized a 103-nucleotide-long intron 1 motif comprising the arrest site and sufficient for obtaining the block in a cell-free transcription assay.

RNA polymerase II usually stops transcription at the ends of genes. However, in certain situations premature termination within coding sequences or introns occurs and serves as a sensitive means for either down-regulating gene expression or generating transcripts with different 3' ends that encode different proteins (for a review, see reference 42). In both cases, neither *cis*- nor *trans*-acting elements required for the arrest of RNA polymerase II and its release from the DNA template were characterized in detail. Concerning transcription arrest at the ends of genes (for a review, see reference 30), investigations have been limited by the very short life span of primary transcripts which are processed at their 3' ends immediately after synthesis. Therefore, transcriptional termination sites have essentially been localized by using run-on assays in isolated nuclei, a technique which allows a rough estimation of RNA polymerases density along genes. Briefly, RNA polymerase II appears to transcribe genes downstream from the polyadenylation signal, sometimes several kilobases further on, and to terminate heterogeneously at multiple arrest sites (16, 19). Premature termination has first been shown to occur within the late transcription unit of simian virus 40 (SV40) (18). Similar observations have been reported for various cellular genes, including *c-myc* (2, 12, 24, 28, 40), *c-fos* (4, 5, 13, 22, 40), *c-myb* (1, 46), *L-myc* (21), and the adenosine deaminase gene (6, 23), as well as in other viral systems such as the early and late transcription units of human adenovirus type 2 (26), mouse polyomavirus (39), minute virus of mice (36), and human immunodeficiency virus (20). Two possibilities may be considered to explain the premature block of transcription elongation. First, RNA polymerase II may be stopped but not released from the DNA template, a process that here will be termed pause. In this case, short abortive RNAs do not accumulate as free molecules. Second, RNA polymerase II is released from the DNA template and liberates short RNAs that accumulate as free molecules unless they are short-lived. This mechanism will be referred to as premature

termination. The two mechanisms likely are not mutually exclusive. In fact, it has been proposed in the case of SV40 that pausing precedes and initiates premature termination (18).

The *c-fos* proto-oncogene encodes a short-lived nuclear phosphoprotein which acts as a transcriptional factor interacting with the members of the Jun family to form the AP1 complex (7, 14, 17, 32, 38). It is subjected to an exquisite regulation involving complex transcriptional and posttranscriptional controls (reviewed in reference 4). Its mRNA and protein products are expressed at low or undetectable levels in most cell types. However, their synthesis can be induced generally very rapidly and transiently by numerous stimuli (for a review, see reference 10). For this reason, it has been proposed that *c-fos* constitutes a ubiquitous master switch that converts short-term stimulations into long-term responses as diverse as differentiation, cell proliferation, and even memory formation (10).

Most studies concerning *c-fos* gene transcription regulation have addressed initiation and have allowed the characterization of several regulatory boxes upstream from the promoter, the most documented one being the serum-responsive element (15, 44, 45). However, studies by our laboratories have pointed to the existence of intragenic regulatory elements. A more intense transcriptional activity was observed in the 5' than in the 3' part of the gene in hamster (13), human (4), mouse (5, 40), and rat (22) cells. Whether the block to transcription elongation is due to pausing or premature termination, this mechanism appears to constitute an important transcriptional regulation level in various situations. As examples, (i) it fine-tunes the transcriptional induction of *c-fos* during the early steps of liver regeneration in the mouse (40), and (ii) in quiescent and exponential mouse Ltk⁻ fibroblasts, although the gene exhibits an important basal level of transcription initiation, only low or undetectable amounts of RNA accumulate because of a strong elongation block presumably acting as a safety lock (5). More recently, a negative intragenic element has been located at the end of the first exon (22). Whether it

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participates in the repression of transcription initiation or elongation has not been determined (see Discussion).

To precisely define sequence motifs involved in the transcription elongation block within the mouse *c-fos* gene, we have developed a cell-free transcription system. Using this assay, we have (i) mapped a unique arrest site 385 nucleotides downstream from the promoter at the level of a T-rich motif that is reminiscent of block sites found in other genes and most likely corresponds to the actual block site previously located by using nuclear run-on assays performed with short DNA probes, (ii) delineated a 103-nucleotide-long intron 1 motif, comprising 75 nucleotides upstream and 28 downstream from the arrest site, sufficient for obtaining the elongation block in vitro (interestingly, RNA synthesized from this region is potentially able to form secondary structures), and (iii) shown that initiation at the *c-fos* promoter is not an absolute requirement for correct transcription arrest within the *c-fos* gene.

MATERIALS AND METHODS

Cell cultures. Mouse fibroblasts (Ltk⁻ cells) were grown at 37°C and in an atmosphere equilibrated with 5% CO₂ in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. Mouse Friend erythroleukemia cells (MEL) (clone 745A; kind gift of G. B. Rossi) were grown in RPMI 1640 medium supplemented with 10% fetal calf serum.

Analysis of *c-fos* gene expression. (i) **Nuclear run-on transcription assay.** After 48 h of serum starvation, Ltk⁻ cells were washed twice with phosphate-buffered saline and lysed with a Dounce homogenizer in 10 volumes of 0.25 M sucrose–10 mM Tris-HCl (pH 8.3)–10 mM NaCl–10 mM MgCl₂–1 mM dithiothreitol–0.5% (vol/vol) Nonidet P-40. Nuclei were purified through a sucrose cushion as previously described (24) and resuspended in 15 mM Tris-HCl (pH 7.4)–15 mM NaCl–60 mM KCl–0.5 mM phenylmethylsulfonyl fluoride–0.5 mM dithiothreitol–0.25 M sucrose–5% (vol/vol) glycerol and stored in liquid nitrogen until use. The nuclear run-on transcription assay was performed essentially as described in reference 40. Briefly, 4 × 10⁷ nuclei were incubated with 200 µCi of [α -³²P]UTP (400 Ci/mmol; Amersham) for 10 min. The reaction was stopped by solvent extraction, and the labeled RNAs were partially hydrolyzed in the presence of 0.3 N NaOH for 5 min at 0°C and hybridized to 5 µg of the appropriate alkali-treated DNA probes immobilized onto nitrocellulose as previously described (24). Double-stranded plasmid DNA was used, since antisense transcription of *c-fos* was not detected in cells of the fibroblastic lineage (13). Hybridization was for 72 h in 50% (vol/vol) formamide–0.75 M NaCl–50 mM sodium phosphate (pH 7)–1 mM EDTA–0.1% sodium dodecyl sulfate (SDS) in the presence of yeast tRNA (100 µg/ml). Stringent washings were conducted at 65°C in 0.1 × SSC (0.15 M NaCl, 0.015 M sodium citrate). The filters were then treated with 10 µg of RNase A per ml in 1 × SSC for 30 min at 37°C before autoradiography.

(ii) **Cell-free transcription assay.** Nuclear extracts were prepared from Ltk⁻ or MEL cells as described previously (11) and stored at –80°C. Protein concentrations were estimated by using the Bio-Rad assay kit. Transcription experiments were performed at 30°C for 60 min in a 30-µl standard reaction mixture containing 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.9)–8 mM MgCl₂–50 mM KCl–0.5 mM phenylmethylsulfonyl fluoride–0.5 mM dithiothreitol–12.5% (vol/vol) glycerol–0.2 mM EDTA–2 mM creatine phosphate–500 µM each of the four

unlabeled ribonucleotides triphosphates, 25 µg of supercoiled DNA template per ml, and 2 to 4 µg of nuclear proteins. RNA products were extracted with an equal volume of phenol-chloroform (1/1) and then precipitated with 2 volumes of ethanol. Pellets were washed twice with 70% ethanol, redissolved in 100 µl of 50 mM Tris-HCl (pH 7.5)–6 mM MgCl₂–2 mM spermidine–10 mM NaCl, and incubated for 15 min at 30°C in the presence of 30 U of RNase-free DNase I (Bethesda Research Laboratories) to degrade the DNA template. RNAs were recovered by phenol extraction, concentrated by ethanol precipitation, and then analyzed in an RNase A protection assay.

Characterization of the in vitro-transcribed RNA products.

(i) **RNase A protection assay.** The *c-fos* *Sma*I-*Xho*I (probe 1; nucleotides –599 to +578) and *Nae*I-*Xho*I (probe 4; nucleotides +41 to +578) restriction fragments, the *c-fos* DNA fragment from PM40.16 (probe 3; nucleotides +313 to +578) and PM37.37 (probe 2; nucleotides –599 to +251) (see plasmid constructions) were cloned into the pBS vector. Radioactive RNA probes were synthesized by using either the T3 or T7 phage RNA polymerase (Genofit) according to the supplier's specification in the presence of 50 µCi of [α -³²P]UTP (400 Ci/mmol) and purified by polyacrylamide gel electrophoresis. In each assay, 50% of the RNA products obtained in a typical transcription experiment were hybridized for 12 h at 45°C in 30 µl of 80% formamide–40 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES; pH 6.8)–400 mM NaCl–1 mM EDTA. Then 300 µl of 300 mM NaCl–10 mM Tris-HCl (pH 7.4)–5 mM EDTA–100 µg of RNase A per ml was added, and the mixture was incubated for 1 h at 30°C. The reaction was stopped by addition of 20 µl of 10% SDS and 100 µg of proteinase K per ml, and the mixture was incubated for 15 min at 37°C. After phenol extraction and ethanol precipitation in the presence of 30 µg of yeast tRNA as carrier, protected RNA fragments were fractionated by electrophoresis through a 5% polyacrylamide gel containing 8 M urea.

(ii) **Primer extension analysis.** A synthetic oligonucleotide primer complementary to the region from +310 to +327 of the mouse *c-fos* transcript was labeled at its 5' end with [γ -³²P]ATP by using T4 polynucleotide kinase. Hybridizations to RNAs synthesized in vitro were carried out at 65°C for 1 h in 10 µl of 40 mM Tris-HCl (pH 7.5)–60 mM KCl–6 mM MgCl₂. Then 80 µl of a solution containing 50 mM Tris-HCl (pH 8.3), 6 mM MgCl₂, 40 mM KCl, 0.5 mM each of the four deoxynucleotide triphosphates, 30 U of RNasin (Genofit), and 2.5 U of avian myeloblastosis virus reverse transcriptase was added. Incubation was performed at 46°C for 30 min. Elongated products were phenol extracted, ethanol precipitated, and fractionated through a 6% polyacrylamide gel containing 8 M urea.

(iii) **Plasmid constructions.** Plasmid P1700.3 was generated by cloning the region of the mouse *c-fos* proto-oncogene extending from position –1100 to +578 into the *Sac*I-*Xho*I restriction sites of the pUC19 vector (a generous gift of B. Verrier). The P75/15 *c-fos* plasmid was a kind gift of U. R  ther (37). The Ad2.fos construct was obtained by subcloning the 4.2-kb *Bam*HI-*Bam*HI restriction fragment of P75/15 (37), which contains the entire murine *c-fos* gene except for the promoter region and the first 41 nucleotides, downstream from the adenovirus type 2 major late promoter into the *Bam*HI site of plasmid PM97 (25) (kind gift from J.M. Egly).

Deletion mutants PM40.01, PM40.07, PM40.08, PM40.14, PM40.16, and PM47.16 were obtained as follows. The *Bam*HI-*Xho*I fragment of the P75/15 *c-fos* construct (spanning nucleotides +41 to +578 of the mouse *c-fos* gene) was

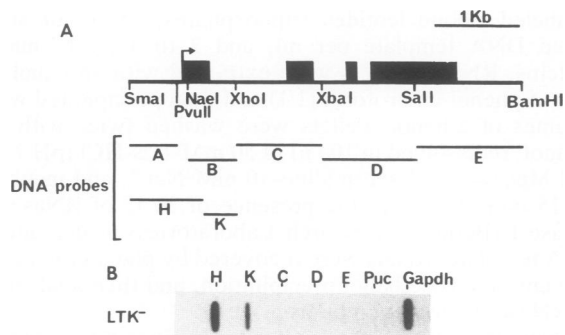


FIG. 1. Run-on analysis of transcription elongation of the *c-fos* proto-oncogene. Nascent RNA transcripts were labeled as described in Materials and Methods. Nuclei were isolated from 48-h serum-starved murine Ltk⁻ fibroblasts. (A) Physical map of the *c-fos* proto-oncogene. Probes A (476 bp), B (536 bp), C (893 bp), D (1,196 bp), and E (1,400 bp) were cloned into the pUC vector by using the indicated restriction sites. H and K were derived from deletion mutants described in Materials and Methods and stretch from nucleotides -499 to +251 and +250 to +578, respectively. The bent arrow marks the transcriptional start site, and solid boxes represent the exons. (B) Run-on assay. The autoradiogram represents the density of RNA polymerase on the *c-fos* transcription unit. Plasmid pUC was introduced to give an estimation of the nonspecific hybridization signal, and the glyceraldehyde-3-phosphate dehydrogenase gene (*Gapdh*) is a positive transcription control.

subcloned into the *Bam*HI-*Sal*I sites of the pBS vector (construct PM39). Plasmid PM39 was cleaved both with *Kpn*I and *Bam*HI restriction enzymes, and controlled digestions with exonuclease III (*Exo*III) were performed. The *Exo*III nuclease-resistant *Kpn*I site (3' protruding) and *Exo*III-degraded region were blunt ended by using S1 nuclease. Plasmids were then recircularized in the presence of T4 DNA ligase and transfected into the RRI strain of *Escherichia coli*. Deletion products were sequenced after cloning into the M13 phage and then cloned downstream from the mouse *c-fos* promoter into the *Eco*RI and *Hind*III sites of plasmid pFL3G (34).

Deletion mutants PM37.02, PM37.07, PM37.10, and PM37.17 were obtained by subcloning the *Hind*III-*Sph*I fragment of P1700.3 in the pBS vector. After cleavage of both *Bam*HI and *Sal*I sites of the pBS cloning polylinker, deletion with *Exo*III was performed as described above and characterized by sequencing. Appropriate mutants were used for in vitro transcription (see Fig. 4A).

RESULTS

The transcriptional elongation block occurs within the first intron of the *c-fos* gene. Using nuclear run-on assays, we have previously reported a higher density of RNA polymerases within the first 580 nucleotides of the mammalian *c-fos* gene, i.e., a region comprising the whole first exon and the 5' moiety of the first intron (4, 5, 13, 22, 40) (probe B; nucleotides +41 to +578; Fig. 1A). To localize more precisely the transcription arrest site, similar experiments were conducted with nuclei purified from exponential and quiescent murine Ltk⁻ fibroblasts because in these situations the *c-fos* gene exhibits an important basal level of transcription initiation and a strong elongation block (see introduction). However, probe B was split here in two roughly equivalent parts relative to the transcribed sequences (Fig. 1A): probe H (nucleotides -499 to +251), which spans the whole exon

1 except the last 39 nucleotides, and probe K (nucleotides +249 to +578), which carries the last 41 nucleotides of exon 1 and part of intron 1. Reproducible hybridization patterns were obtained whether exponential or quiescent cells were used. Results of a typical experiment using quiescent Ltk⁻ cells (Fig. 1B) show that radioactive signals are detectable on both probes H and K but are undetectable on the more downstream C, D, and E probes. This finding indicates that transcription terminates very close to the 3' end of exon 1 or within the first intron. Uridine contents of probes H and K being comparable, the H/K radioactive signal ratio of 2 to 3, as deduced from densitometer scanning of autoradiographs, allows us to estimate a mean length of nearly 300 to 400 nucleotides for nascent RNA transcripts and thus locates the transcriptional block site within the first third of intron 1.

Localization of a unique transcription arrest site 385 nucleotides downstream from the mouse *c-fos* promoter. Run-on experiments are neither sensitive nor precise enough to determine whether RNA polymerase stops randomly, as is usually the case at the ends of genes (30), or at discrete sites, as has been shown in the case of premature termination within the *c-myc* gene (8). To address this issue, an in vitro transcription assay using nuclear extracts from Ltk⁻ fibroblasts and MEL cells (line 745A) according to the method of Dignam et al. (11) was developed. Plasmid P1700.3, which carries a region of the mouse *c-fos* proto-oncogene extending from position -1100 to position +578 (Fig. 2D), was used as a template to generate in vitro transcripts. If transcription elongation was blocked at unique sites, discrete RNA species shorter than the predicted 578-nucleotide-long runoff transcript should be easily characterized. Conversely, randomly terminated RNAs should appear as a smear. Transcription experiments were performed in the presence of unlabeled triphosphate nucleotides, and in vitro-synthesized RNAs were subsequently characterized by RNase mapping using uniformly labeled RNA probes (see Materials and Methods and legend to Fig. 2D).

In addition to Ltk⁻ fibroblasts, MEL cell extracts were used because we have previously used them to characterize the correct transcription block site within the murine *c-myc* gene (to be published elsewhere), thus suggesting that the transcription termination machinery was active. The analysis of transcription products revealed a minor 578-nucleotide-long transcript corresponding to the full-length RNA species and a shorter major protected fragment of approximately 380 nucleotides (a typical experiment performed with MEL cell extracts is presented in Fig. 2A). These results suggest that (i) although not offering definitive proof of what happens in vivo, our in vitro assay is able to reproduce efficiently the elongation block observed in isolated nuclei, (ii) the elongation block occurs at a unique site (see below) within the 5' part of intron 1, and (iii) the location of the arrest site is not peculiar to a given cell type. Both Ltk⁻ and MEL cell extracts were therefore used in the following experiments.

To rule out possible in vitro transcription artifacts, two controls were performed. First, transcription experiments were carried out in the presence of a low concentration of α -amanitin (2 μ g/ml). This condition is known to inhibit specifically RNA polymerase II. The data presented in Fig. 2A clearly demonstrate that in vitro transcription initiated at the *c-fos* promoter is RNA polymerase II dependent. This observation is in full agreement with the inhibition of *c-fos* transcription observed in run-on assays in the presence of 2 μ g of α -amanitin per ml (data not shown). Second, to verify that the 380-nucleotide-long RNA transcript initiates at the

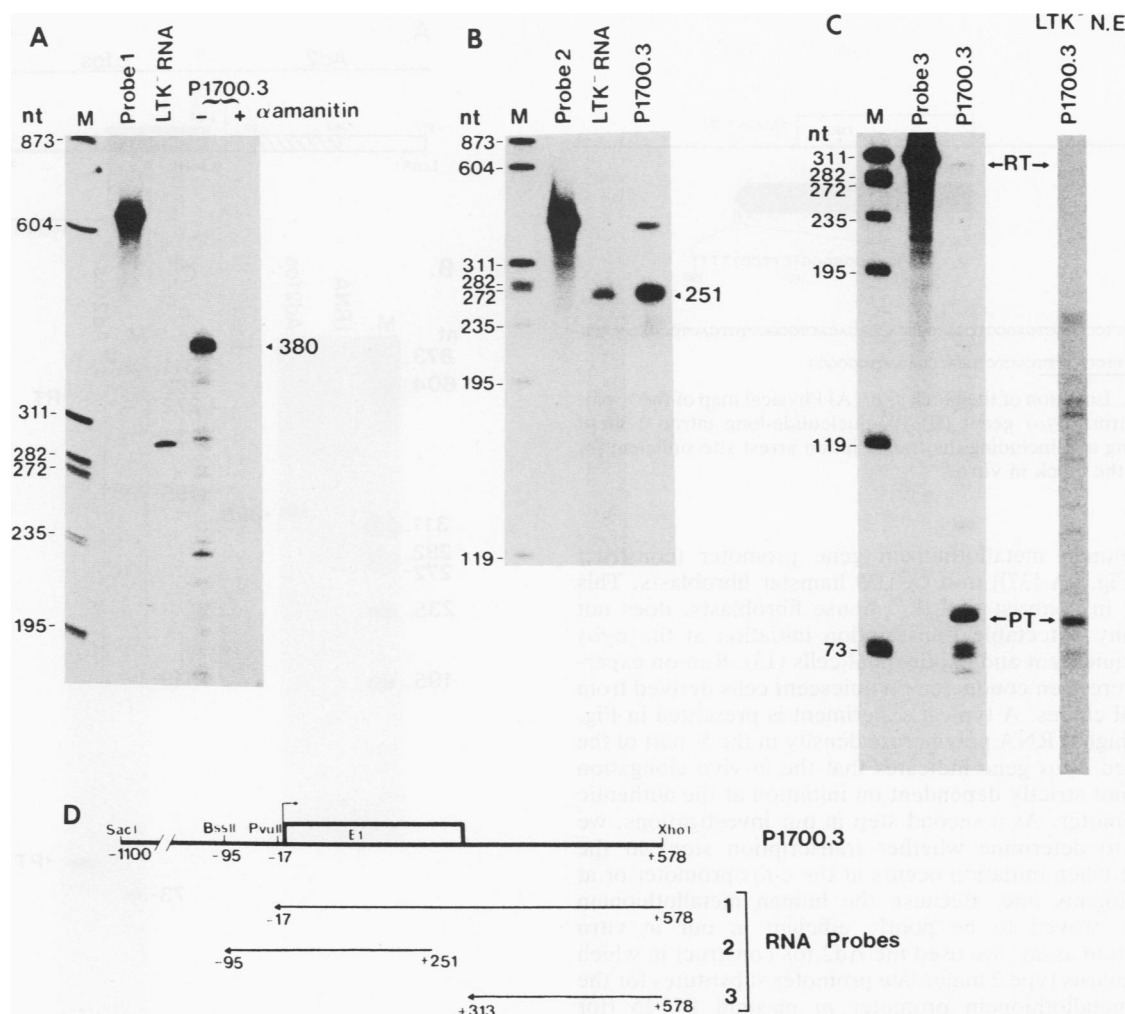


FIG. 2. In vitro transcription of the mouse *c-fos* gene, using nuclear protein extracts from MEL cells. All reactions were performed as described in Materials and Methods. (A) RNAs synthesized in vitro from the P1700.3 construct were compared in an RNase A protection assay using probe 1 with 10 μ g of total RNAs prepared from 30-min serum-stimulated quiescent Ltk⁻ cells (Ltk⁻ RNA). Transcriptions were performed without (P1700.3 -) or with (P1700.3 +) α -amanitin (2 μ g/ml). The solid arrow at position 380 indicates prematurely terminated transcripts. Size markers (M) are *Hae*III-digested bacteriophage ϕ X174 replicative-form DNA labeled with terminal transferase by using [α -³²P]ddATP. (B) Mapping of the 5' end of *c-fos* transcripts generated in vitro. RNAs from 30-min serum-stimulated Ltk⁻ cells (Ltk⁻ RNA) and in vitro-transcribed RNA from the P1700.3 construct were hybridized to the uniformly labeled probe 2. After RNase A digestion, protected fragments were fractionated through a 6% polyacrylamide sequencing gel. The size of the protected fragment (251 nucleotides [nt]) is indicated. (C) Determination of the 3' end of *c-fos* transcripts generated in vitro by using nuclear protein extracts from MEL cells and Ltk⁻ fibroblasts (Ltk⁻ N.E). The RNase A protection assay was performed by using probe 3, which is derived from construct PM40.16 (see Materials and Methods). Protected fragments are indicated. PT and RT refer to prematurely terminated and readthrough transcripts, respectively. (D) Physical map of the plasmid P1700.3 and locations of the different RNA probes. Restriction sites and ends of each probe are indicated. The open box represents the first exon of the mouse *c-fos* gene, and bent arrow marks the initiation site.

normal *c-fos* gene promoter, RNAs generated in vitro were compared in an RNase protection assay with the *c-fos* mRNA accumulating in quiescent Ltk⁻ cells stimulated for 30 min with serum. To this end, RNAs were mapped with probe 2 (Fig. 2D). As shown in Fig. 2B; in both situations, a 251-nucleotide-long protected fragment, consistent with initiation at the authentic *c-fos* promoter, was observed. This finding suggests that transcription initiates correctly in our in vitro assay.

Finally, the 3' end of the short RNA transcript was mapped more precisely with an RNA probe spanning the region of the block site (probe 3; Fig. 2D). Experiments performed with both Ltk⁻ and MEL cell extracts are pre-

sented in Fig. 2C. A 75-nucleotide-long protected fragment (PT in Fig. 2C) allowed us to locate precisely the block site at position +385 relative to the cap site. Remarkably, the transcription block site lies in a G-rich region (for nucleotide sequences, see Fig. 3) at the level of a T-rich stretch reminiscent of other situations (see Discussion).

The premature elongation block is not dependent on the *c-fos* promoter. To determine whether the transcription arrest is strictly dependent on either initiation at the *c-fos* promoter or the effect of sequences located upstream from the latter, we have selected a two-step approach. In a first set of experiments, we transfected a plasmid carrying a chimeric mouse *c-fos* gene under the transcriptional control

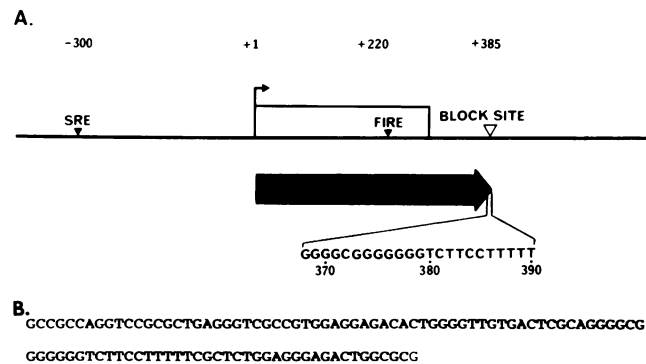


FIG. 3. Location of the block site. (A) Physical map of the 5' part of the murine *c-fos* gene; (B) 103-nucleotide-long intron 1 motif surrounding and including the transcription arrest site sufficient for obtaining the block in vitro.

of the human metallothionein gene promoter (construct P75/15; Fig. 4A [37]) into CCL39 hamster fibroblasts. This cell line, in contrast to Ltk⁻ mouse fibroblasts, does not exhibit any detectable transcription initiation at the *c-fos* locus in quiescent and exponential cells (13). Run-on experiments were then conducted on quiescent cells derived from individual clones. A typical experiment is presented in Fig. 4B. The higher RNA polymerase density in the 5' part of the transfected *c-fos* gene indicates that the in vivo elongation block is not strictly dependent on initiation at the authentic *c-fos* promoter. As a second step in our investigations, we planned to determine whether transcription stops at the same site when initiation occurs at the *c-fos* promoter or at a heterologous one. Because the human metallothionein promoter proved to be poorly efficient in our in vitro transcription assay, we used the Ad2.fos construct in which the adenovirus type 2 major late promoter substitutes for the human metallothionein promoter in plasmid P75/15 (for details, see legend to Fig. 5A and Materials and Methods). 3' ends of RNAs generated from Ad2.fos were characterized by RNase mapping with probe 3 (Fig. 2C); 5' ends were mapped by reverse transcription using a synthetic oligonucleotide hybridizing to the region extending from nucleotides

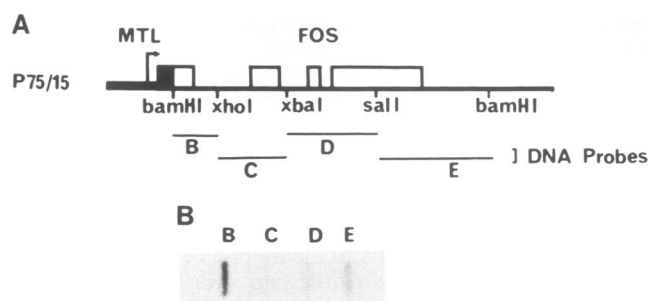


FIG. 4. Run-on analysis of P75/15 *c-fos* vector expression in transfected CCL39 cells. Nuclei from CCL39 fibroblasts transfected with the P75/15 construct were purified, and nascent RNA transcripts were labeled as described in Materials and Methods. (A) Physical map of P75/15. The thick line and the solid box represent the metallothionein gene promoter (MTL); open boxes represent mouse *c-fos* gene exons; the thin line represents introns and 3' downstream sequences. Double-stranded DNA probes are the same as in Fig. 1A. (B) Autoradiogram of the run-on assay carried out on the chimeric *c-fos* gene.

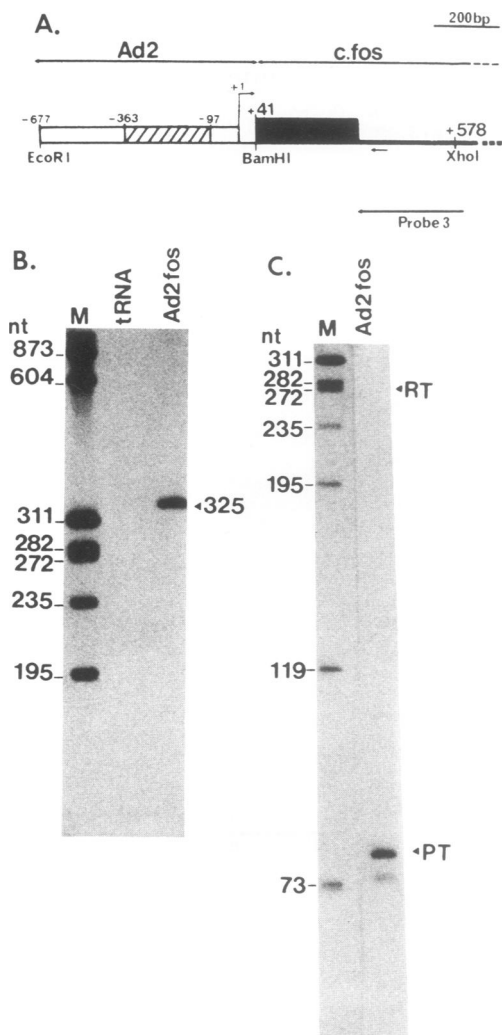


FIG. 5. In vitro transcription of the *c-fos* gene under the control of the adenovirus type 2 major late promoter (Ad2.fos). (A) Physical map of Ad2.fos. Ad2 (open box) refers to a mutated adenovirus type 2 major late promoter deleted from the region spanning nucleotides -97 to -363 (hatched box) (plasmid PM97) (25); the solid box and the thick line represent the *c-fos* gene; the short arrow represents the synthetic oligonucleotide complementary to nucleotides +310 to +327 used for primer extension analysis; the long arrow represents the antisense RNA probe 3. (B) Primer extension analysis. The oligonucleotide was 5' end labeled with the T4 polynucleotide kinase and hybridized to RNAs generated in vitro from the chimeric Ad2.fos construct. A similar reaction was conducted with 10 μ g of yeast tRNA as a negative control. The reverse-transcribed products were separated through a sequencing gel. The cDNA size is indicated (325 nucleotide [nt]). (C) 3' end analysis of the in vitro-synthesized RNAs. The RNase A protection assay was conducted with probe 3 (Fig. 2C). PT and RT represent prematurely terminated and readthrough transcripts, respectively.

+310 to +327. In both experiments, radioactive bands of the expected size were obtained (Fig. 5B and C). This finding demonstrates that transcription from the Ad2.fos construct actually initiates at the adenovirus type 2 promoter and that the elongation block occurs at the same site as when transcription starts at the *c-fos* promoter. In conclusion, both run-on and in vitro transcription experiments suggest

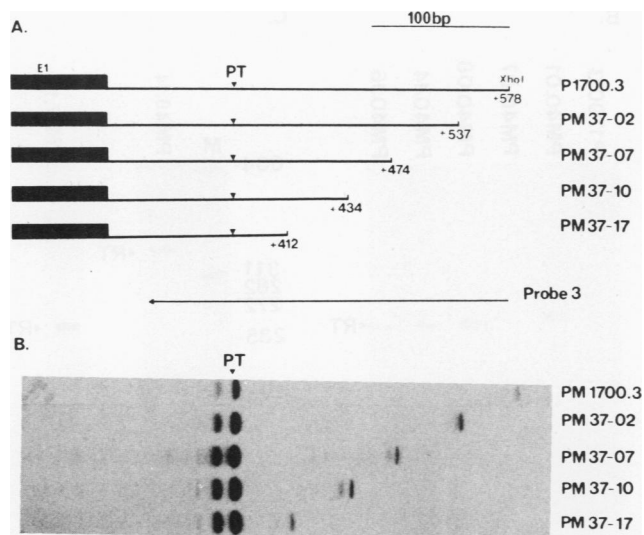


FIG. 6. In vitro transcription of mutants deleted downstream from the block site. (A) Physical maps of the mutants analyzed. The solid box represents the *c-fos* exon 1; PT represents the location of the block site. 3' ends of deletions are indicated relative to the cap site. (B) Comparison of in vitro transcripts generated from each construct with those derived from P1700.3 in an RNase A protection assay using probe 3 (Fig. 2C). Protected RNA fragments were fractionated through a 6% polyacrylamide sequencing gel. PT represents the prematurely terminated transcripts.

that the *c-fos* promoter region is not required for transcription arrest within *c-fos* intron 1.

Sequences downstream from the arrest site are not necessary for obtaining the elongation block. We next addressed the possibility that sequences located downstream of the transcription arrest site are involved in regulation of the elongation block. For this purpose, a series of deletion mutants was generated by using ExoIII. In all constructs, the promoter region and the 5' part of the *c-fos* gene extending down to the transcription arrest site remain unaltered. All deletions begin at the *XhoI* site located at position +578 and extend 5' toward the arrest site. Mutants PM37.02, PM37.07, PM37.10, and PM37.17 were selected for transcription experiments. They end at positions +412, +434, +474, and +537, respectively, relative to the cap site (Fig. 6A; Materials and Methods). Transcripts generated in vitro were characterized by RNase mapping with probe 3 (Fig. 2C). In all situations, RNAs begin at the *c-fos* promoter (data not shown) and terminate prematurely at position +385 (Fig. 6B). This demonstrates that the transcriptional elongation block occurring in vitro within *c-fos* intron 1 does not depend on sequences located downstream from the arrest site, except perhaps the 28 nucleotides just downstream from the latter.

Seventy-five nucleotides upstream from the arrest site are sufficient for obtaining the elongation block. The minimal sequence upstream from the arrest site allowing the block of transcription elongation in our in vitro assay was also delineated. To this end, a series of mutants harboring deletions beginning at the *c-fos* promoter and extending toward the arrest site was generated. The promoter region spanning nucleotides -499 to -17, which thus includes the TATA box but not the cap site, was conserved in all mutants, as well as sequences located downstream from the transcription arrest site as far as nucleotide +578. Deletions

were generated by ExoIII nuclease degradation. The multiple cloning steps required to derive the expression vectors are described in Materials and Methods. Since the normal initiation site is deleted, transcription starts within the cloning polylinker used to adapt internal *c-fos* gene sequences onto the *c-fos* promoter. Mutants PM40.01, PM40.07, PM40.08, PM40.14, PM40.16, and PM47.16, deleted from position -17 to nucleotide +40, +100, +134, +250, +313, and +330, respectively, were selected for in vitro transcription experiments. RNA products generated in transcription assays were characterized by RNase mapping using probes 3 and 4 (Fig. 7A). Clones fall into two categories (Fig. 7B). In the first category are mutants as block efficient as plasmid P1700.3, which include PM40.01, PM40.07, PM40.08, PM40.14, and PM40.16 (Fig. 7B). However, in several experiments performed with different nuclear extract preparations, the elongation block within the PM40.16 mutant is more or less efficient (data not shown). Whether this results from the preparation of the nuclear extract itself or from subtle modifications of cell metabolism has not been investigated. The second category consists in block-inefficient mutants, since only the runoff RNA is detected regardless of the nuclear extract tested; it is represented by the PM47.16 construct (Fig. 7C). These observations indicate that the 75 nucleotides located upstream from the arrest site are sufficient to reproduce in vitro the elongation block. However, the fact that high levels of readthrough transcription are sometimes detected in the case of PM40.16 raises the possibility that different elements cooperate for efficient transcription arrest within *c-fos* intron 1.

DISCUSSION

We have previously shown that a block to transcription elongation occurs, at the most, 578 nucleotides downstream from the initiation site and may contribute to the down-regulation of the mammalian *c-fos* gene in both cultured cells (4, 5, 13, 22) and animal tissues (40). In this study, we have mapped a putative transcription block site and delineated in a cell-free transcription assay the sequences required for efficient transcription arrest in the mouse *c-fos* gene.

Location of the transcription block site. Two approaches were used to map precisely the site at which transcription stops in the 5' part of the mouse *c-fos* proto-oncogene. First, using a transcription assay in isolated murine fibroblast nuclei, we have roughly located the arrest site within the first third of *c-fos* intron 1, 300 to 400 nucleotides downstream from the promoter. A similar observation has been made recently in mouse macrophages (9a). Then, using a cell-free transcription system, we have confirmed this observation and shown that RNA polymerase II either pauses or prematurely terminates transcription at a discrete site 385 nucleotides downstream from the *c-fos* promoter. This situation differs from the heterogeneous termination of transcription observed at the ends of most genes (30). On the contrary, it is reminiscent of the premature termination involved in the regulation of other cellular and viral genes (8, 18, 31, 41). Unfortunately, we have not been able to definitely prove that the arrest site mapped in vitro actually corresponds to the one used in vivo, since characterization of short abortive RNAs turned out to be impossible with use of a sensitive RNase protection assay (see below). Moreover, we were unable to develop a quantitative polymerase chain reaction analysis since priming of reverse transcriptase with oligonucleotides complementary to the G/C region situated upstream from the putative stop site turned out to be inefficient

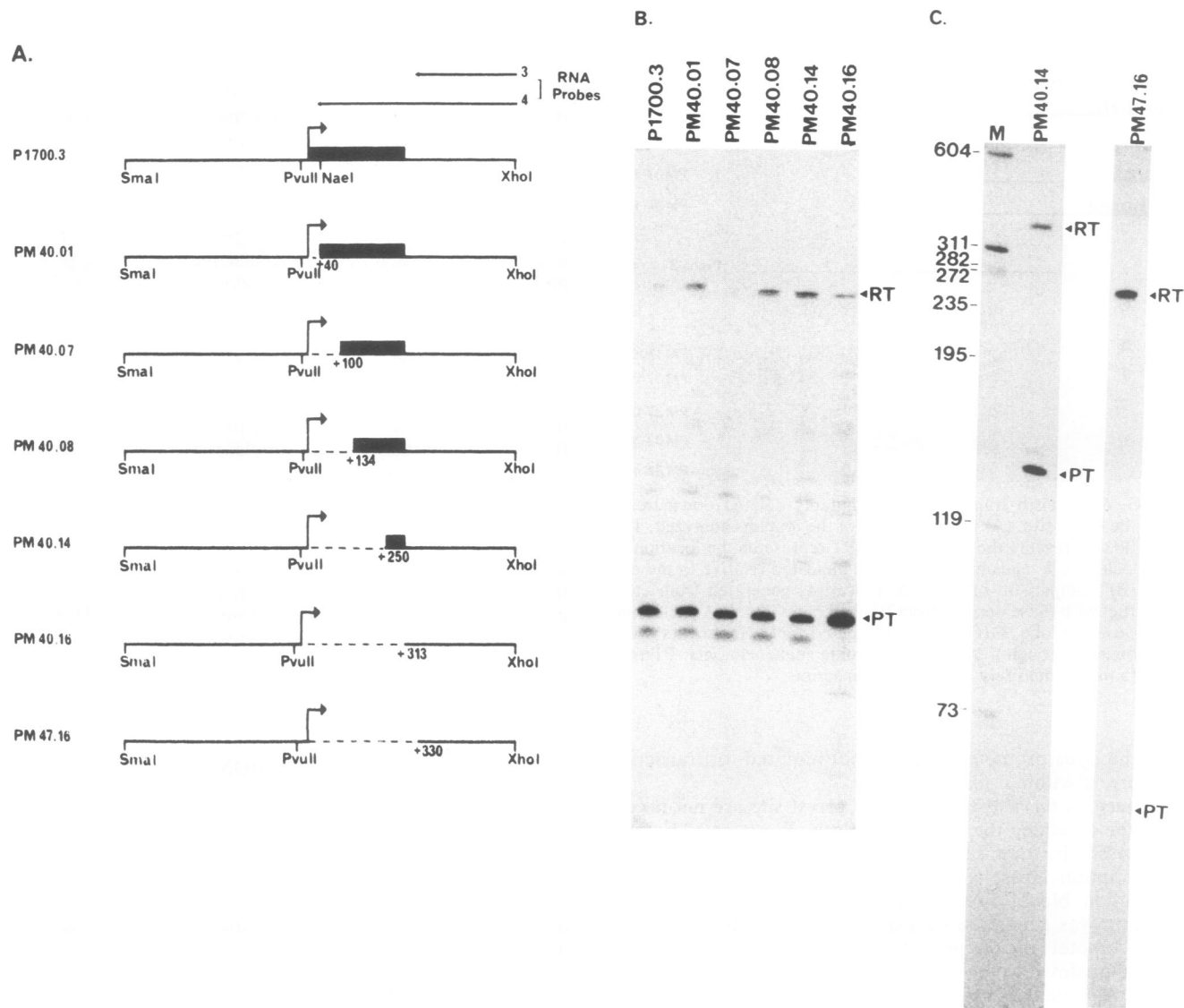


FIG. 7. In vitro transcription of mutants deleted upstream from the block site. (A) Physical maps of PM40.01, PM40.07, PM40.08, PM40.14, PM40.16, and PM47.16 deletion mutants. The solid box represents *c-fos* exon 1, the arrow represents the promoter, and the dashed lines represent deletions. All of the deletions begin at position -17 relative to the cap site and end at the positions indicated. (B) Analysis of in vitro RNA generated from P1700.3, PM40.01, PM40.07, PM40.08, PM40.14, and PM40.16 by RNase mapping using probe 3 (Fig. 2C). (C) Analysis of in vitro products generated by PM40.14 and PM47.16, using probe 4 (Fig. 7A). Protected fragments were fractionated through a 6% polyacrylamide sequencing gel. PT and RT refer to prematurely terminated and readthrough transcripts, respectively. Sizes are indicated in nucleotides.

(data not shown), probably because of the potential for this region to form secondary structures (see below).

Promoter dependence. It has been shown that correct transcription termination at the end of U1 and U2 small nuclear RNA genes requires the association of proteins with RNA polymerase II at specific motifs located upstream from the transcription initiation site (9, 29). Similarly, it has been suggested that premature termination within the *c-myc* gene preferentially affects transcripts initiated at the P2 3'-most promoters (43; our unpublished data). Such a promoter effect was tested by substituting the human metallothionein gene promoter (construct P75/15) or the major late adenovirus type 2 promoter (construct Ad2.fos) in place of the normal *c-fos* promoter. Run-on assays performed on cells transfected with plasmid P75/15, and in vitro transcription

experiments carried out with the Ad2.fos construct clearly showed that termination within *c-fos* intron 1 is not strictly dependent on its homologous promoter. This observation supports the idea that intragenic motifs per se can be responsible for basal levels of transcription elongation block but do not exclude the possibility that modulation is determined at the initiation level under specific conditions. Such a situation is observed in the case of the *c-myc* proto-oncogene, since (i) the efficiency of the elongation block appears to be correlated to the use of the P2 promoter in the normal *c-myc* gene (see above); (ii) an internal 190-nucleotide-long region of the mouse *c-myc* exon 1 is able to promote premature termination in intact cells when it is inserted within the rabbit α -globin gene, which does not exhibit any transcription elongation block per se (47); (iii)

replacement of the human *c-myc* promoter by the human thymidine kinase gene promoter conserves the elongation block in *Xenopus laevis* oocytes (3); and (iv) and the mouse *c-myc* gene under the control of the adenovirus type 2 major late promoter also conserves the elongation block in our in vitro transcription assay (to be published elsewhere).

Intragenic sequence requirements for efficient transcription block. Transcription arrest at a discrete site strongly supports the idea that structural motifs at either the DNA or RNA level are responsible for the inhibition of transcription elongation. Moreover, modulation of the block in various physiological situations calls for the existence of *trans*-acting factors able to recognize the putative *cis*-acting determinants. When tested in our cell-free transcription assay, deletions upstream and downstream from the transcription arrest site allowed us to delineate a 103-nucleotide-long motif, comprising 75 nucleotides upstream and 28 nucleotides downstream from the arrest site, that gives rise to the transcription block (Fig. 3). Experiments are under way in our laboratory to demonstrate that this sequence motif is sufficient to reproduce the elongation block in vivo. However, our experimental data do not allow us to discriminate whether the sequences are important at the DNA or RNA level. Nevertheless, several points concerning this region are worth mention. First are transcription stops close to a T-rich element (Fig. 3). This situation is reminiscent of the pause sites characterized in several prokaryotic genes and the premature termination sites observed in several viral and eukaryotic genes (42). However, although probably favoring transcription termination, the T-rich element is not an absolute requirement for in vitro premature termination within the *c-myc* gene. Along this line, no such element exists in the vicinity of the premature termination site of the adenosine deaminase gene (31). Site-specific mutagenesis will be needed to address this issue in the case of the mouse *c-fos* gene. Second, a stable hairpin structure ending just upstream from the T run can be deduced from the nucleotide sequence (Fig. 8). Such structures have been proposed to play a major role in the control of attenuation in prokaryotic genes and of premature termination in various viral and cellular genes (12, 18, 31, 41). Site-directed mutagenesis will be needed to demonstrate its involvement in *c-fos* gene transcription regulation. Third, it has been recently shown in our laboratory that a negative *c-fos* intragenic regulatory element (FIRE) situated at the end of exon 1 (Fig. 3) is involved in negative regulation of the rat *c-fos* gene (22). Its biological effect was characterized through microinjection into living cells of double-stranded DNA fragments acting as putative competitors for DNA-binding proteins. However, its biochemical role has not yet been determined. Both run-on and in vitro transcription experiments presented here indicate that FIRE is clearly distinct from the elongation block site itself, since the latter lies nearly 150 nucleotides downstream from it within intron 1. In addition to revealing a negative regulatory element when inserted alone into a β -galactosidase gene under the transcriptional control of the mouse *c-fos* promoter, this element does not appear to be an absolute requirement for inducing the elongation block, since it is absent in block-efficient mutants PM40.14 and PM40.16 (Fig. 7A). Although not definitively excluding its participation in modulation of the transcription block, our data argue for its involvement in the control of transcription initiation. In conclusion, the existence of discrete transcription arrest sites in regions exhibiting structural similarities raises the hypothesis that processes of transcription elongation inhibition occurring in various higher eukaryotic genes,

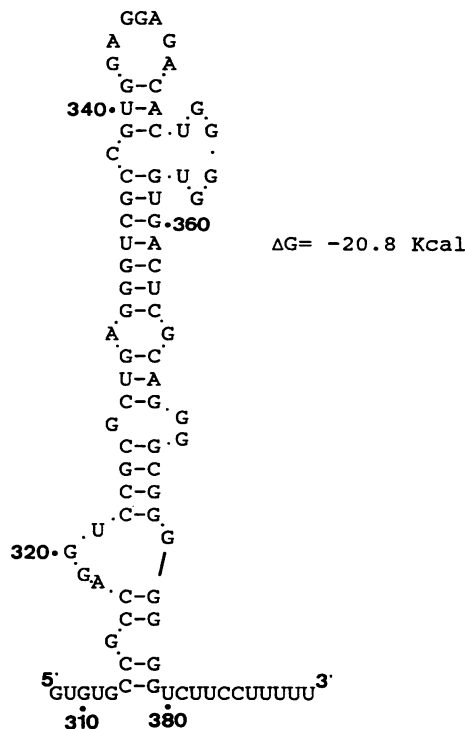


FIG. 8. Hypothetical secondary RNA structure ending just upstream from the arrest site. Shown is a model of the secondary structure of the 75-nucleotide-long block-efficient sequence located upstream from the block site as predicted by the algorithm of Zuker and Stiegler (48); $-20.8 \text{ kcal} = \text{ca. } -87 \text{ kJ}$.

including the *c-fos* proto-oncogene, share some common mechanisms. However, it is already clear that modulation of this regulation involves different pathways, since these genes are usually expressed in different physiological conditions (42).

Pausing or premature termination within the *c-fos* gene?

Two lines of observation indicate that premature termination might be the mechanism responsible for the transcription block in viral and higher eukaryotic genes. First, using *X. laevis* oocytes as a transcription assay, Bentley and Groudine (3) have suggested that premature termination is responsible for the elongation block within the *c-myc* gene. This hypothesis has recently received further support from the characterization of an abortive RNA transcript, which accumulates during establishment of the transcriptional block in human promyelocytic HL60 cells induced to differentiate into granulocytes (33). Second, premature termination has been proposed to be the ultimate mechanism that actually controls the expression of the late transcription unit of SV40 (18). However, the same authors have shown that pausing, likely promoted by a hairpin structure, precedes transcription termination and thus suggest that the two processes may be related. To determine whether premature termination is the mechanism actually involved in *c-fos* gene repression, we have performed sensitive RNase protection and Northern (RNA) blotting experiments to detect and to quantify accumulation of abortive RNAs in Ltk⁻ cells. However, in none of these experiments were we able to characterize such species. This suggests either that our assays are not sensitive enough to detect RNAs resulting from pausing or that prematurely terminated RNAs are very unstable, as is usu-

ally the case for *c-myc*, except in differentiating HL60 cells (33). Use of the *X. laevis* oocyte transcription system, which exhibits low RNase activity, and analysis of various physiological situations in which *c-fos* gene transcription elongation is strongly inhibited will help to resolve this important issue.

Which kind of *trans*-acting factors for modulating the elongation block? *trans*-acting factors involved in the transcription elongation block may interact with either DNA or RNA motifs. Two lines of evidence support each possibility. On one side, it has recently been shown that the Tat protein is able to bind newly synthesized RNAs for modulating the elongation block occurring within the long terminal repeat of human immunodeficiency virus (41). It has also been reported that secondary structures of nascent RNA transcripts might be instrumental in relieving the block within the SV40 (18), *c-myc* (12, 24), and adenosine deaminase (31) genes, thereby suggesting interactions with RNA-binding proteins. On the other hand, variations in the binding of proteins to specific DNA motifs have been correlated with modulations of the elongation block occurring within *c-myc* gene (35). Using *in vitro* gel retardation and footprinting assays, we have recently shown that a protein(s) can specifically recognize a *c-fos* intron 1 DNA motif that includes part of the U-rich stretch of the RNA polymerase II arrest site and belongs to the 103-nucleotide-long region defined as sufficient for transcription elongation inhibition (to be published elsewhere). However, we have not yet been able to characterize variations in binding efficiency. Genomic footprinting on living cells will help to determine whether the same complex is formed *in vivo* and whether subtle modifications occur as the block varies.

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